MOLECULAR AND CELLULAR BIOLOGY, Mar. 1993, p. 1392–1407
0270-7306/93/031392-16$02.00/0

Vol. 13, No. 3

Activation of Heat Shock Gene Transcription by Heat Shock Factor 1 Involves Oligomerization, Acquisition of DNA-Binding Activity, and Nuclear Localization and Can Occur in the Absence of Stress

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Received 17 September 1992/Returned for modification 20 November 1992/Accepted 3 December 1992

The existence of multiple heat shock factor (HSF) genes in higher eukaryotes has prompted questions regarding the functions of these HSF family members, especially with respect to the stress response. To address these questions, we have used polyclonal antisera raised against mouse HSF1 and HSF2 to examine the biochemical, physical, and functional properties of these two factors in unstressed and heat-shocked mouse and human cells. We have identified HSF1 as the mediator of stress-induced heat shock gene transcription. HSF1 displays stress-induced DNA-binding activity, oligomerization, and nuclear localization, while HSF2 does not. Also, HSF1 undergoes phosphorylation in cells exposed to heat or cadmium sulfate but not in cells treated with the amino acid analog L-azetidine-2-carboxylic acid, indicating that phosphorylation of HSF1 is not essential for its activation. Interestingly, HSF1 and HSF2 overexpressed in transfected 3T3 cells both display constitutive DNA-binding activity, oligomerization, and transcriptional activity. These results demonstrate that HSF1 can be activated in the absence of physiological stress and also provide support for a model of regulation of HSF1 and HSF2 activity by a titratable negative regulatory factor.

The induction of heat shock gene expression by heat and other forms of stress has been studied for several decades as a paradigm for inducible gene expression (25). The activation of heat shock gene transcription during the stress response is mediated by heat shock transcription factor (HSF), which binds to heat shock elements (HSE) in the promoters of heat shock genes (2, 4, 30, 56, 57). In the yeast *Saccharomyces cerevisiae*, HSF is bound to DNA in unstressed cells and undergoes heat shock-dependent phosphorylation concomitant with transcriptional competence (18, 42, 45). In unstressed *Drosophila* and vertebrate cells, however, HSF exists in a non-DNA-binding form which is rapidly converted to the DNA-binding form by elevated temperature and other stresses, such as treatment with cadmium sulfate or the amino acid analog L-azetidine-2-carboxylic acid (20, 27, 30, 43, 56, 59). This conversion is accompanied by oligomerization (53). In addition, human HSF from heat-shocked cells has been shown to be phosphorylated (22).

Genes encoding HSF have been isolated from the yeasts *S. cerevisiae* and *Kluyveromyces lactis* and from fruit fly, tomato, human, mouse, and chicken cells (8, 19, 29, 33, 35–37, 45, 54). The yeasts *S. cerevisiae* and *K. lactis* and the fruit fly *Drosophila melanogaster* each have a single HSF gene. A recent, somewhat unexpected development has been the finding that a number of species, including tomato, human, mouse, and chicken, contain multiple HSF genes.

The presence of multiple HSFs in a single species has raised an interesting question. Are all of these HSFs involved in regulating the stress response? Their classification as HSFs stems from their ability to specifically bind HSE-containing sequences. In addition, sequence comparisons of these proteins from different species demonstrate strong homology in their DNA-binding and oligomerization domains (33, 35–37). However, the two mouse HSFs, HSF1 and HSF2, have only 38% identity overall (35), and with the exception of DNA-binding activity, little is known about their functional properties. One functional difference that we have identified is that mouse HSF1 translated in vitro in a rabbit reticulocyte lysate displays heat-inducible DNA-binding activity, whereas mouse HSF2 binds DNA constitutively (35). These results are consistent with a potential role for HSF1 in the activation of heat shock gene transcription in response to stress.

To determine the functional roles of HSF1 and HSF2 in regulating the stress response, we raised antibodies specific to these factors and used them to characterize the physical, biochemical, and biological properties of these two factors in both unstressed and stressed mouse and human cells. We have found that HSF1 is the primary component of the HSF DNA-binding activity present in cells exposed to heat shock, cadmium sulfate, or the amino acid analog L-azetidine-2-carboxylic acid. HSF1 undergoes stress-induced oligomerization, acquisition of DNA-binding activity, and localization to the nucleus, while HSF2 does not. Interestingly, HSF1 exhibits an increase in phosphorylation after exposure to heat or cadmium sulfate but not after treatment with azetidine. This finding suggests that an increase in phosphorylation of HSF1 is not absolutely required for oligomerization, DNA-binding activity, nuclear translocation, or transcriptional activity of this factor. HSF1 and HSF2 overexpressed in 3T3 cells display constitutive oligomerization, DNA-binding activity, and transcriptional activity. Overexpressed HSF1 is localized exclusively to the nucleus, while overexpressed HSF2 is present in both the cytoplasm and nucleus. These results demonstrate that HSF1 can be activated in the absence of stress and provide support for the existence of a titratable negative regulator which functions to control the activity of HSF1 and HSF2.

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MATERIALS AND METHODS

Expression of mouse HSF1 and HSF2 in *Escherichia coli* and antibody production. Open reading frames for full-length mouse HSF1 and HSF2 were placed behind a bacterial T7 promoter in the pET-3A vector (49) after polymerase chain reaction-mediated mutagenesis of the respective start codons to create an *Nde*I site. The pET-mHSF1 and pET-mHSF2 constructs were transformed into *E. coli* BL21(DE3) host cells, overexpression of HSF1 and HSF2 was induced, and extracts were prepared as described previously (49). The overexpressed HSF1 and HSF2 proteins were run on preparative sodium dodecyl sulfate (SDS)-polyacrylamide gels, excised, and electroeluted. Rabbits were immunized with mouse HSF1 or HSF2, using 500 μg of protein in complete Freund's adjuvant for the primary injection followed by three boosts of 250 μg in incomplete Freund's adjuvant at 4-week intervals. Serum was prepared from the rabbits from blood taken 10 days after the fourth injection of antigen, and this serum was used for all experiments.

Western immunoblot analysis. Whole cell extracts (15 to 40 μg) or bacterially expressed mouse HSF1 and HSF2 were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose, using a Bio-Rad semidry transfer apparatus according to the manufacturer's protocol. For phosphatase treatment, 20 to 30 μg of whole cell extract was incubated with 1 U of potato acid phosphatase (type VII; Sigma) with or without ammonium molybdate (10 μM, final concentration) at 37°C for 60 min before being subjected to SDS-PAGE. After blocking for 90 min with phosphate-buffered saline (PBS) containing 2.5% nonfat dry milk, filters were incubated for 60 min at 22°C with a 1:10,000 dilution of the HSF1 or HSF2 antiserum. Filters were washed three times for 10 min each time with PBS–0.1% Tween 20 and incubated for 45 to 60 min with a 1:40,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (Boehringer Mannheim). Blots were washed six times for 5 min each time with PBS–0.1% Tween 20 and detected by using the ECL system (Amersham). For quantitation of molecules of HSF1 and HSF2 in 3T3 and HeLa cells, whole cell extracts from known numbers of cells were subjected to Western blot analysis with known amounts of purified recombinant HSF1 or HSF2. Calculations were based on theoretical molecular weights of 54,800 and 57,273 for mouse and human HSF1, respectively, and 58,160 and 60,347 for mouse and human HSF2, respectively.

For the pore exclusion limit electrophoresis, whole cell extracts were run on 3 to 17% polyacrylamide gels containing 1× TBE (90 mM Tris-borate, 2 mM EDTA) with a 3 to 15% glycerol gradient for 2,800 V-h at 4°C, and the gels were processed as described previously (5, 53) before being subjected to Western blot analysis. For native molecular size standards, a mixture of thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), and bovine serum albumin (BSA; 69 kDa) was run in a separate lane and visualized after Western blot analysis with amido black. Linear regression analysis of native marker molecular weights was performed, and a line was obtained with an r^2 value of 0.988.

Cross-linking of HSF was performed by adding 1/10 volume of dimethyl sulfoxide containing appropriate concentrations of EGS [ethylene glycol bis(succinimidylsuccinate); Pierce] to whole cell extracts from unstressed or heat-shocked cells and then incubating the cells at 22°C for 30 min. Reactions were quenched by addition of glycine to 75 mM and subjected to SDS-PAGE (5% resolving gels), and the HSF cross-linked products were visualized by probing with the HSF1 antiserum.

Native gel shift analysis. Cells were heat shocked and whole cell extracts were prepared as previously described (27). Extracts from *E. coli* overexpressing mouse HSF1 or HSF2 were prepared by incubating bacterial pellets in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–10% glycerol–100 mM KCl–0.2 mM EDTA at 22°C for 10 min and then subjecting them to quick freezing in a dry ice–methanol bath. After thawing at 22°C, cell debris was pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C. Supernatants were quick-frozen and stored at −80°C. A native gel shift assay was performed as described previously (26) with a self-complementary ideal HSE oligonucleotide (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′) which contains four perfect inverted NGAAN repeats when annealed. For experiments involving addition of antibodies to whole cell extracts prior to gel shift analysis, 1 μl of either preimmune serum (1:10 dilution) or immune serum (1:10, 1:50, or 1:250) was added to 2 μl of whole cell extract (10 μg of protein) and incubated at 22°C for 20 min before assays for DNA binding.

Immunofluorescence analysis. Immunofluorescence analysis was performed essentially as described previously (24). Fixation with methanol, ethanol, or acetone gave essentially identical results. The HSF1 and HSF2 antisera were each used at a dilution of 1:300, with 1:1,500 dilutions of the hsp70 monoclonal antibody 3A3 (28). Rhodamine-conjugated goat anti-rabbit immunoglobulin G and fluorescein-conjugated goat anti-mouse immunoglobulin G (Cappel) were used at dilutions of 1:700 after preadsorption with fixed 3T3 and HeLa cells.

Transfection of 3T3 cells with HSF1 and HSF2 expression vectors. For overexpression of mouse HSF1 and HSF2 in mammalian cells, the full-length *Eco*RI cDNA inserts of each were blunt ended by filling in the overhanging ends and then ligating them to the blunt-ended *Bam*HI site of the β-actin-1-neo expression vector such that they would be under the control of the β-actin promoter (17). hsp70-luciferase was created by subcloning a 350-bp *Bam*HI fragment containing 188 bp of the proximal human hsp70 promoter into the *Bgl*II site of pJD205 (9). The transfections were done by using calcium phosphate precipitation as described previously (12). Mouse 3T3 cells were seeded at a density of 500,000 per plate and transfected with 10 μg of plasmid DNA per plate the following day. The cells were incubated overnight at 37°C, and fresh medium was added after removal of the DNA precipitate by washing the cells once with PBS containing 3 mM EGTA and then once with PBS. After 24 h, the cells were harvested for whole cell extracts or fixed for immunofluorescence as described above. Luciferase and chloramphenicol acetyltransferase assays were performed as previously described (9, 16).

RESULTS

Constitutive DNA-binding activity of HSF1 and HSF2 expressed in *E. coli*. The mouse HSF1 and HSF2 proteins were overexpressed in *E. coli* by using a T7 polymerase-based expression system (49). Since HSF1 and HSF2 in unstressed mammalian cells do not bind DNA, gel shift analysis was performed with extracts from bacteria that overexpress HSF1 or HSF2 to determine whether their DNA-binding activity would also be negatively regulated in *E. coli*. As shown in Fig. 1, both HSF1 and HSF2 expressed in *E. coli* bind DNA constitutively. Induction of HSF1 and HSF2

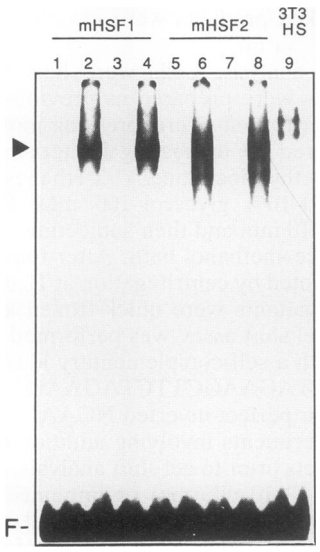

FIG. 1. Constitutive DNA-binding activity of HSF1 and HSF2 expressed in *E. coli*. Extracts of *E. coli* containing a mouse HSF1 or HSF2 (mHSF1 or mHSF2) expression plasmid were examined for HSE-binding activity by a native gel shift assay either before (lanes 1 and 5) or after (lanes 2 and 6) isopropylthiogalactopyranoside (IPTG) induction. Specificity of DNA binding was determined by competition with an unlabeled HSE oligonucleotide (lanes 3 and 7) or an oligonucleotide containing a CCAAT binding site (lanes 4 and 8). The HSE-binding activity present in an extract of heat-shocked (HS) 3T3 cells is shown in lane 9.

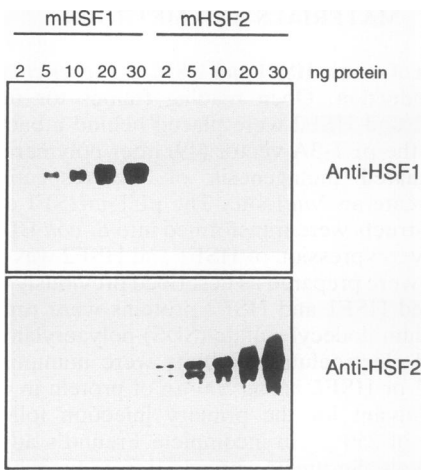

FIG. 2. Characterization of the specificity of HSF1 and HSF2 antisera. Purified bacterially expressed mouse HSF1 and HSF2 (mHSF1 and mHSF2) were run on SDS-polyacrylamide gels and subjected to Western blot analysis using the HSF1 antiserum (upper panel) or HSF2 antiserum (lower panel) at a dilution of 1:10,000. The amount of HSF1 or HSF2 loaded in each lane is indicated. The faster-migrating band in the lanes probed with the HSF2 antiserum is known to be a degradation product of HSF2 (data not shown).

expression in *E. coli* gives rise to HSE-binding activities (lanes 2 and 6) which are specifically competed for by the HSE oligonucleotide (lanes 3 and 7) but not by an oligonucleotide containing a CCAAT element (lanes 4 and 8). No HSE-binding activity is observed in uninduced *E. coli* extracts (lanes 1 and 5). The constitutive DNA-binding activity displayed by the bacterially expressed HSF1 and HSF2 has been previously observed for recombinant *Drosophila* HSF and human HSF1 and HSF2 (8, 33, 37).

Characterization of the specificity of the HSF1 and HSF2 antisera. To characterize the biochemical and biological properties of mouse HSF1 and HSF2, rabbit polyclonal antisera were raised against SDS-PAGE-purified recombinant HSF1 and HSF2. The antisera were first assayed for reactivity to purified recombinant HSF1 and HSF2 by enzyme-linked immunosorbent assay. Both antisera were found to be specific for their respective antigens, at dilutions of up to 1:20,000 (34). To determine whether the antisera would specifically recognize the native forms of HSF1 and HSF2, immunoprecipitations were performed with in vitro-translated mouse HSF1 and HSF2. The HSF1 and HSF2 antisera reacted strongly with HSF1 and HSF2, respectively, but showed no reactivity with the other antigen (34).

Western blot analysis was also performed with purified recombinant HSF1 and HSF2 to determine whether the antisera would specifically recognize the respective antigens by this method. The HSF1 antiserum reacted strongly with purified HSF1 (Fig. 2, upper panel) but showed no visible cross-reactivity with HSF2 even with the highest amount tested (30 ng). Likewise, the HSF2 antiserum reacted strongly with HSF2 but displayed no cross-reactivity with HSF1 (lower panel). Therefore, in three different assays, these antisera have been shown to react specifically with their respective antigens.

Heat shock-induced HSF is composed of HSF1. The cloning of multiple HSF genes from several higher eukaryotes raises questions regarding the functions of these proteins, especially with respect to their roles in the stress response. To begin to address these questions, we sought to determine whether the HSE-binding activity induced by heat shock is composed of HSF1 or HSF2 or both. One method for determining the composition is to perform gel shift analysis in which extracts from heat-shocked cells are preincubated with the HSF1 and HSF2 antisera. Specific recognition by the antisera should result either in neutralization of DNA-binding activity or decreased mobility of the HSF-DNA complex as a result of the association of several antibody molecules. Antibodies directed against *Drosophila* HSF have previously been shown to recognize HSF in the gel shift assay (58).

To test the feasibility of this approach, the antisera were first tested to determine whether they could specifically recognize native recombinant HSF1 and HSF2 in conjunction with the gel shift assay. Figure 3A shows that the HSF1 and HSF2 antisera specifically recognize the native DNA-binding forms of *E. coli*-produced HSF1 (upper panel) and HSF2 (lower panel), respectively, even at dilutions of up to 1:250. A slight amount of cross-reactivity is observed for both antisera at high antibody concentrations (1:10 dilution). The amount of bacterially produced HSF1 or HSF2 used in this assay (0.1 ng) was adjusted to approximate the amount of HSF1 or HSF2 present in extracts of mammalian cells (see below). Identical results were obtained by using mouse HSF1 and HSF2 translated in rabbit reticulocyte lysate (34).

These antisera were then used to examine whether HSF1 or HSF2 or both are present in the HSE-binding activities of heat-shocked mouse 3T3 cells and human HeLa cells. The results shown in Fig. 3B demonstrate that HSF1 is the primary component of the heat-induced HSE-binding activities of both 3T3 cells (upper panel) and HeLa cells (lower panel). The HSF1 antiserum recognizes the HSE-binding activities even at relatively low antibody concentrations

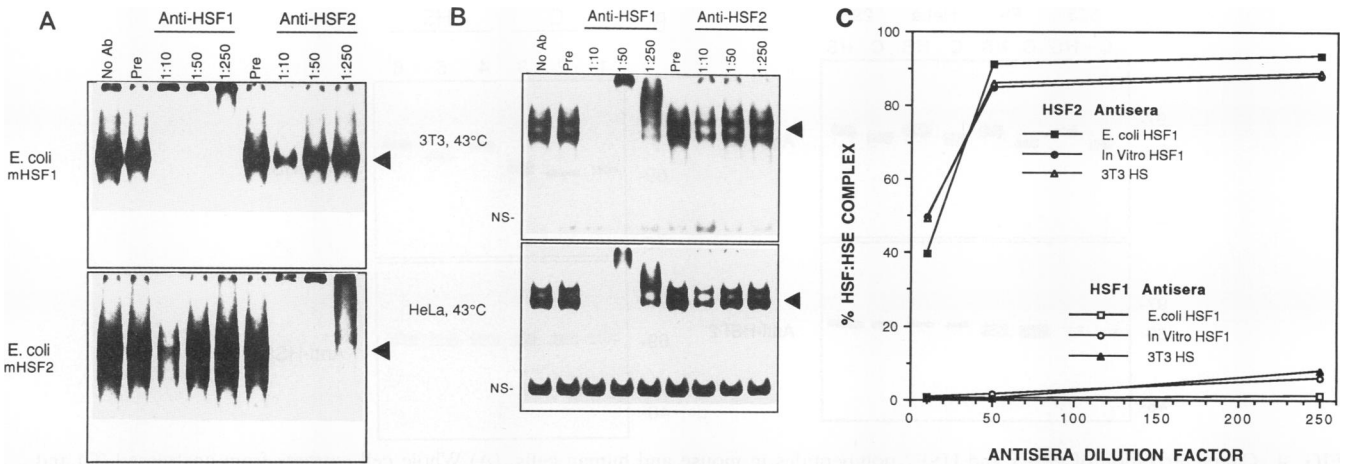

FIG. 3. Antibody recognition of native recombinant or natural HSF. (A) Extracts containing 0.1 ng of recombinant HSF1 (upper panel) or HSF2 (lower panel) produced in *E. coli* were incubated either alone (no antibody [Ab]), with a 1:10 dilution of preimmune serum (Pre), or with a 1:10, 1:50, or 1:250 dilution of the HSF1 or HSF2 antiserum before being subjected to gel shift analysis. Specific HSF-DNA complexes are indicated by triangles. (B) Whole cell extracts from heat-shocked 3T3 and HeLa cells were incubated with the HSF1 or HSF2 antiserum as for panel A. Specific HSF-DNA complexes (triangles) are indicated along with nonspecific protein-DNA complexes (NS). (C) HSF-DNA complexes from gels in panels A and B and from antibody recognition gels using in vitro-translated recombinant HSF1 or HSF2 (data not shown) were quantitated by scanning laser densitometry and plotted as percentages of maximal HSF-DNA complex (no antibody added).

(1:250 dilution), while the HSF2 antiserum has an effect only at high concentrations (1:10 dilution). This pattern is very similar to that observed for pure recombinant HSF1 (Fig. 3A). The effects of the HSF1 and HSF2 antisera on the HSE-binding activity from heat-shocked mouse 3T3 cell extracts (Fig. 3B) were quantitatively compared with their effects on bacterially produced (Fig. 3A) or in vitro-translated recombinant HSF1 (34) by scanning the HSF-DNA complexes with a densitometer and plotting the results as a percentage of maximal (no antibody added) HSE-binding activity (Fig. 3C). This comparison clearly shows that the recognition of HSE-binding activity from heat-shocked cells by the HSF1 and HSF2 antisera is essentially identical to their recognition of recombinant, bacterially produced HSF1. These results demonstrating that HSF1 is the primary component of heat-induced HSE-binding activity indicate that HSF1 and HSF2 have distinct functional roles.

Characterization of HSF1 and HSF2 polypeptides in mammalian cells. HSF exists in unstressed cells in a latent, non-DNA-binding form which is converted to a DNA-binding form by exposure of cells to stress. While it has been possible to study the DNA-binding form of HSF, analysis of the non-DNA-binding form has been hampered by the inability to detect it. The generation of polyclonal antibodies specific to HSF1 and HSF2 has allowed us for the first time to examine the physical and biochemical properties of HSF1 and HSF2 from unstressed cells and to compare these properties directly with those of HSF1 and HSF2 present in heat-shocked cells. To examine HSF1 and HSF2 expression in different cell types and to determine whether they are subject to heat shock-dependent modification, Western blot analysis was performed with extracts from unstressed and heat-shocked mouse and human cell lines.

Figure 4A shows that both HSF1 and HSF2 are present in mouse and human cells in the absence of stress. The cross-reactivity of the antisera with human HSF1 and HSF2 is not surprising given the large degree of homology between mouse and human HSF1 and between mouse and human HSF2 (90 and 95%, respectively). HSF1 in unstressed mouse and human cells consists of a group of closely sized polypeptides with a size range of approximately 70 to 74 kDa (Fig. 4A, upper panel), which is consistent with the sizes determined for both in vitro-translated and *E. coli*-expressed mouse and human HSF1 (33, 34, 35). After a 120-min, 42°C heat shock, HSF1 exhibits a significant increase in molecular size to approximately 85 to 89 kDa in all of the mouse and human cell lines tested. These results are consistent with previous observations on the size of HSF from heat-shocked human cells (15, 22).

The HSF2 antiserum recognizes two to three discrete polypeptides in extracts from both unstressed and heat-shocked mouse cells, with molecular sizes ranging from 67 to 77 kDa (Fig. 4A, lower panel). The multiple HSF2 polypeptides observed may be produced by alternative splicing of the HSF2 mRNA or posttranslational modification of the HSF2 protein. In the extract of heat-shocked 3T3 cells run on this gel, there is an apparent increase in the amount of the largest HSF2 polypeptide, but this phenomenon is not consistently observed, and its significance is unclear. The distribution of the HSF2 polypeptide subspecies varies between the mouse 3T3 and F9 cell lines. F9 embryonic carcinoma cells appear to have significantly more of the largest and smallest of these polypeptides relative to 3T3 cells. In contrast to mouse HSF2, human HSF2 consists of a very closely migrating doublet with molecular masses of 79 to 80 kDa. The size of HSF2 in mouse and human cells does not change upon heat shock (Fig. 4, lower panel).

To determine whether HSF1 or HSF2 is phosphorylated in unstressed cells and whether the heat-induced increase in molecular weight of HSF1 is due to phosphorylation (Fig. 4A, upper panel), extracts of unstressed and heat-shocked mouse 3T3 cells were treated with potato acid phosphatase and subjected to Western blot analysis. The results in Fig. 4B show that HSF1 in unstressed cells is phosphorylated, because after treatment with phosphatase, the size decreases from a set of polypeptides of 70 to 74 kDa to a predominant species of approximately 70 kDa (compare lanes 1 and 2). The decrease in molecular size is due to dephosphorylation

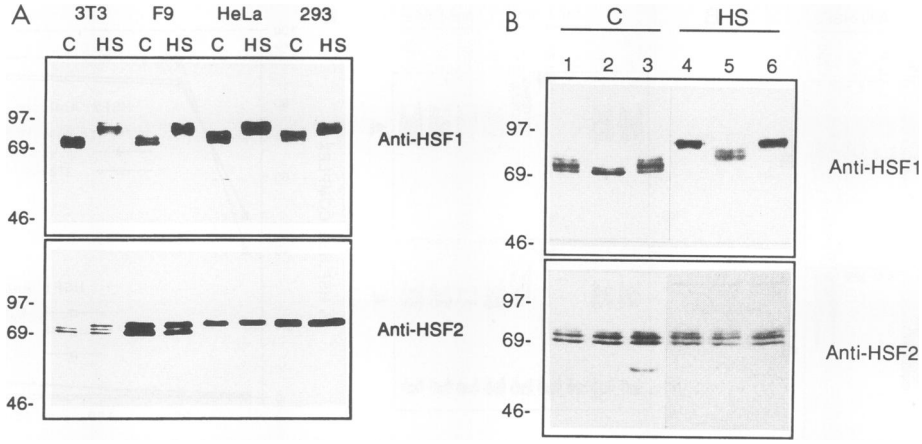

FIG. 4. Characterization of HSF1 and HSF2 polypeptides in mouse and human cells. (A) Whole cell extracts from unstressed (C) and heat-shocked (HS) mouse 3T3 and F9 and human HeLa and 293 cells were run on SDS-polyacrylamide gels and subjected to Western blot analysis using the HSF1 antiserum (upper panel) or HSF2 antiserum (lower panel) at a dilution of 1:10,000. Sizes of molecular mass markers (in kilodaltons) are indicated to the left of each panel. (B) Whole cell extracts from unstressed (lanes 1 to 3) and heat-shocked (lane 4 to 6) 3T3 cells were incubated at 37°C for 60 min either with no treatment (lanes 1 and 4), after treatment with potato acid phosphatase (lanes 2 and 5), or after phosphatase treatment in the presence of ammonium molybdate (lanes 3 and 6) and subjected to Western blot analysis using the HSF1 antiserum (upper panel) or HSF2 antiserum (lower panel).

and not proteolysis, because addition of the phosphatase inhibitor ammonium molybdate to the reactions prevents a decrease in the size of HSF1 (lane 3).

The increase in the size of HSF1 observed upon heat shock is due to phosphorylation, as demonstrated by a decrease in molecular size upon phosphatase treatment from 85 to 89 kDa to 74 to 77 kDa (compare lanes 4 and 5). These results show that HSF1 present in heat-shocked cells is more phosphorylated (an approximately 11-kDa decrease upon phosphatase treatment) than is HSF1 present in unstressed cells (maximum decrease of 4 kDa). However, the size of phosphatase-treated heat-shocked HSF1 is still greater than that of the phosphatase-treated HSF1 in unstressed cells. One explanation is that phosphorylation alone may not account for all of the molecular size change between HSF1 in unstressed and heat-shocked cells. Alternatively, there may be additional phosphates on HSF1 in heat-shocked cells that are not accessible to phosphatase because of the oligomeric nature of activated HSF1 (see Fig. 5). These results are consistent with previous studies which demonstrated that HSF is phosphorylated in stressed yeast and human cells (22, 43). In contrast to HSF1, the size of HSF2 in both unstressed and heat-shocked cells was not altered by phosphatase treatment, suggesting that HSF2 is not phosphorylated (Fig. 4B, lower panel). However, it is important to note that the absence of a mobility change on SDS-polyacrylamide gels is not an absolute measure of changes in the phosphorylation state. Therefore, we cannot unequivocally state that HSF2 is not phosphorylated prior to or after heat shock treatment. Identical results were obtained in experiments on HSF1 and HSF2 from HeLa cells (34).

The results of the Western blot analysis shown in Fig. 4A suggested that the amounts of HSF1 and HSF2 may vary in different mouse and human cell types. To investigate this possibility and to determine whether these amounts change after heat shock, the numbers of molecules of HSF1 and HSF2 per cell were quantitated by comparing the amounts of HSF1 and HSF2 present in unstressed and heat-shocked 3T3 and HeLa extracts with known amounts of purified recombinant HSF1 and HSF2 by Western blot analysis. Calculations based on these quantitations indicate that unstressed mouse 3T3 cells and human HeLa cells contain approximately 16,000 (±1,100) and 30,000 (±3,300) molecules of HSF1, respectively, and 5,300 (±800) and 14,400 (±300) molecules of HSF2 per cell, respectively. After a 120-min, 42°C heat shock, the levels of HSF1 in both 3T3 and HeLa cells do not significantly change from the levels in unstressed cells (14,400 ± 1,500 and 30,000 ± 400, respectively). However, the levels of HSF2 after heat shock decrease somewhat in 3T3 cells and even more significantly in HeLa cells (3,500 ± 1,100 and 8,600 ± 200, respectively). The difference in the effect of heat shock on the levels of HSF1 versus HSF2 may be a reflection of stabilization of the HSF1 protein by heat shock treatment (perhaps mediated by its oligomeric state; see Fig. 5).

Heat shock-induced oligomerization of HSF1. To determine whether HSF1 or HSF2 undergoes changes in native size upon heat shock in vivo, extracts from unstressed and heat-shocked 3T3 cells were run on pore exclusion limit electrophoresis gels and subjected to Western blot analysis (Fig. 5A). This analysis shows that HSF1 in unstressed mouse cells is present as two distinct native sizes. The predominant species comigrates with the BSA marker (69 kDa) and presumably represents monomeric HSF1. A second, less prevalent species has an estimated native size of 360 kDa. Following a 120-min, 42°C heat shock, HSF1 forms a complex which migrates as a diffuse band of very large native size (700 to 800 kDa), which is similar to the sizes observed for *Drosophila* HSF in cytoplasmic extracts exposed to heat in vitro (53) and recombinant HSF1 (34). The complete absence of the presumptive monomer in the heat-shocked cell extracts suggests that upon heat shock, all of the HSF1 in the cell is converted to the higher-molecular-weight, oligomeric form. In contrast, HSF2 exists in unstressed cells predominantly as a species with an approximate native size of 360 kDa and is unaffected by heat shock. It should be noted that the estimated native sizes of HSF1 and HSF2 presented above are based on comparisons with globular protein standards. Since the native structures of HSF1 and HSF2 have not been determined, these numbers

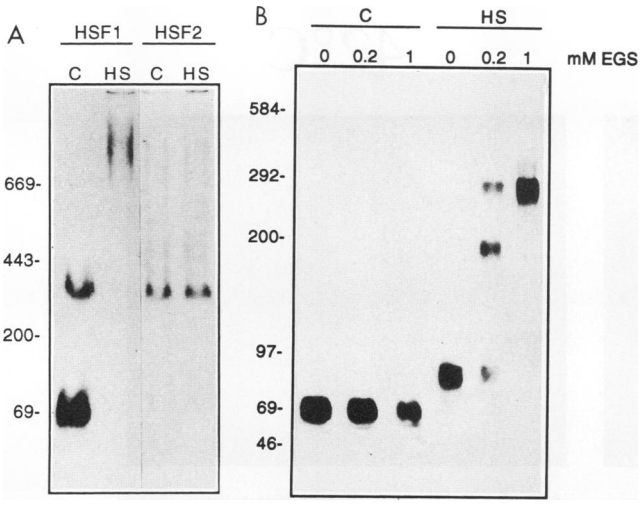

FIG. 5. Native sizes of HSF1 and HSF2 in unstressed and heat-shocked cells. (A) Whole cell extracts from unstressed (C) and heat-shocked (HS) 3T3 cells were run on pore exclusion limit electrophoresis gels and then subjected to Western blot analysis using the HSF1 or HSF2 antiserum. (B) Whole cell extracts from unstressed (C) or heat-shocked (HS) 3T3 cells, either untreated (0 mM) or in the presence of the cross-linking reagent EGS at a final concentration of 0.2 or 1 mM, were run on an SDS-polyacrylamide gel and subjected to Western blot analysis using the HSF1 antiserum.

are not meant to imply a rigorous determination of native complex size and are given primarily to illustrate changes in native size.

As an independent means to confirm the change in native complex size of HSF1 upon heat shock, and to determine the stoichiometry of the heat-activated HSF1 complex, whole cell extracts from unstressed and heat-shocked cells were cross-linked with EGS and then subjected to Western blot analysis using the HSF1 antiserum (Fig. 5B). In extracts of unstressed cells treated with EGS, HSF1 is detected predominantly as a species which comigrates with the BSA marker (69 kDa). On longer exposures, a second species of approximately 140 kDa is also faintly detected (34). These results suggest that the predominant form of HSF1 in unstressed cells is a monomer, which is consistent with the results of the pore exclusion limit analysis.

In heat-shocked cell extracts treated with a low concentration of EGS (0.2 mM), three multimeric forms are detected. The species of highest mobility has a size of approximately 90 kDa and presumably represents monomeric, activated HSF1, while the two lower-mobility species with estimated sizes of 180 and 270 kDa may represent dimers and trimers, respectively, of HSF1. The use of higher concentrations of EGS (1 mM) produced only the 270-kDa cross-linked product. These results suggest that HSF1 in heat-shocked mouse cells exists as a trimer in solution. Similar results have been obtained in cross-linking studies of yeast and fruit fly HSF (32, 44). Cross-linking of HSF2 was not as efficient as for HSF1 but did show the presence of a species with an approximate molecular size of 140 kDa in extracts of both unstressed and heat-shocked cells, suggesting that HSF2 may exist as a dimer (34).

The results of both the pore exclusion limit analysis and the cross-linking studies demonstrate that HSF1 exists predominantly as a monomer in unstressed cells and is converted to an oligomer by heat treatment, while the native size of HSF2 is unaffected by heat shock. The obvious discrepancy between the native sizes of HSF1 and HSF2 measured by pore exclusion limit electrophoresis versus SDS-PAGE analysis of cross-linked proteins may be explained by the fact that these two techniques measure native molecular weight under different conditions. One possibility is that native HSF1 and HSF2 exist as extended, nonglobular structures that differ from the globular protein standards used to estimate size by pore exclusion limit electrophoresis and so may behave as if they have sizes much greater (700 to 800 kDa and 360 kDa, respectively) than the sizes obtained for the cross-linked species on SDS-polyacrylamide gels (270 and 140 kDa, respectively).

Intracellular localization of HSF1 and HSF2 in unstressed and heat-shocked 3T3 and HeLa cells. Previous studies have demonstrated that HSF DNA-binding activity can be generated in vitro by treating cytoplasmic extracts from unstressed mammalian and *Drosophila* cells with heat, biochemical agents, or even anti-HSF antibodies, suggesting that the inactive form of HSF is present in the cytoplasm prior to heat shock (22, 26, 58). However, recent immunocytochemical studies in *Drosophila* S2 cells showed that HSF is localized to the nucleus both before and after heat shock (53). To determine the intracellular localization of HSF1 and HSF2 in mammalian cells, double immunofluorescence was performed on mouse 3T3 fibroblasts and human HeLa cells, using the HSF1 and HSF2 antisera and the hsp70 monoclonal antibody 3A3 (28).

In the absence of stress, HSF1 is distributed in a diffuse pattern in the cytoplasm and nucleus of 3T3 and HeLa cells (Fig. 6A and 6B, upper panels). However, in cells heat shocked at 42°C for 120 min, HSF1 is found primarily in the nucleus of both 3T3 and HeLa cells. The relocalization of HSF1 to the nucleus upon heat shock is consistent with a role as the factor responsible for heat-induced transcription of heat shock genes. The staining patterns in 3T3 and HeLa cells are specific for HSF1, because preincubation of the antisera with purified HSF1 but not HSF2 blocked these patterns (34). Staining of the same cells with the hsp70 monoclonal antibody 3A3 demonstrates that the cells were heat shocked. hsp70 is primarily localized to the cytoplasm in unstressed cells, while in heat-shocked cells, the characteristic translocation of hsp70 to nucleoli is observed (Fig. 6) (31, 52).

There is a distinct difference in the HSF1 staining pattern between heat-shocked 3T3 and HeLa cells. While there is diffuse HSF1 staining throughout the nucleus in both cell types, distinct, brightly staining granules are observed in heat-shocked HeLa cells but not in 3T3 cells (Fig. 6B, upper panel). There are four to six granules per nucleus, with a maximum size of 2 to 2.5 μm. These granules have been observed in a number of human cell lines but not in any of several mouse cell lines examined, suggesting that the presence of these granules may represent a characteristic difference between human and mouse cells (34). These punctate structures do not correspond to nucleoli as observed by phase microscopy and hsp70 staining of the nucleoli by the hsp70 monoclonal antibody 3A3 (34) (Fig. 6B).

The results of the immunofluorescence analysis on the intracellular localization of HSF1 were confirmed by Western blot analysis of cytoplasmic and nuclear fractions of unstressed and heat-shocked mouse 3T3 cells. Figure 7 (upper panel) shows that HSF1 is present in both the cytoplasm and nucleus of unstressed cells, while the phos-

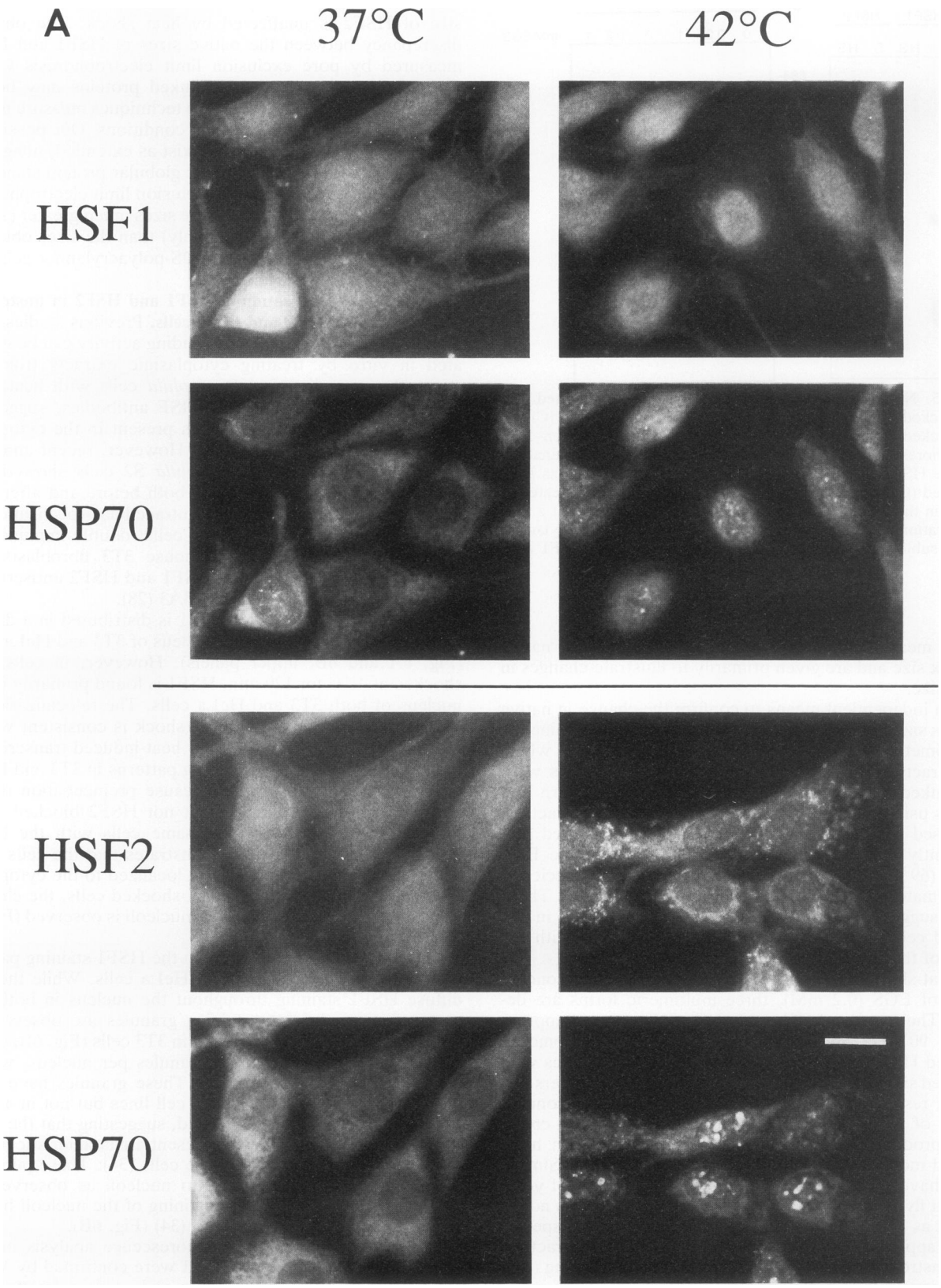

FIG. 6. Intracellular localization of HSF1 and HSF2 in unstressed and heat-shocked 3T3 and HeLa cells. (A) Unstressed and heat-shocked 3T3 cells were subjected to double immunofluorescence analysis using the HSF1 or HSF2 antiserum and the anti-hsp70 monoclonal antibody 3A3. (B) Immunofluorescence analysis of unstressed and heat-shocked HeLa cells was performed as for panel A. Bars correspond to 15 μm.

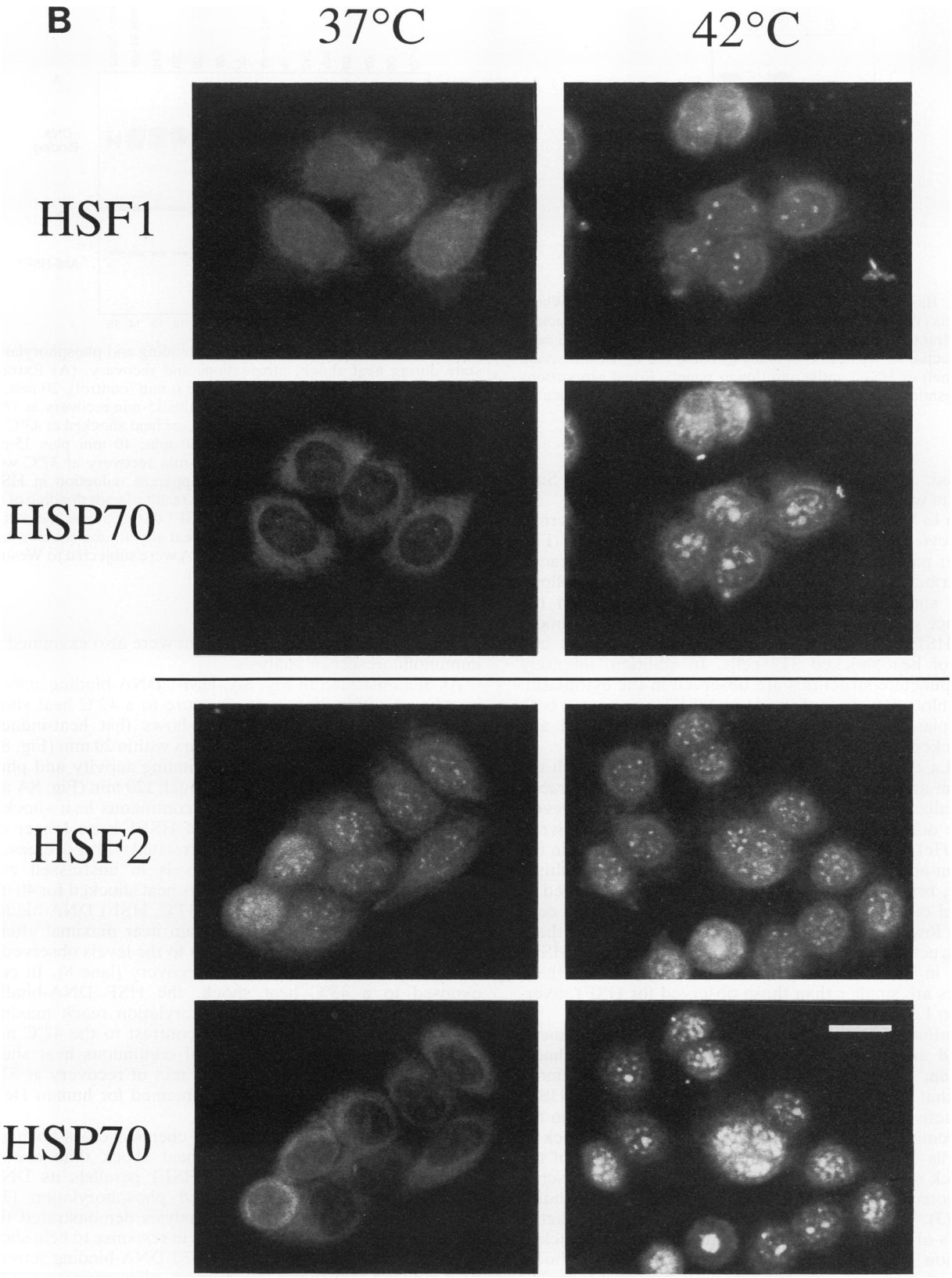

FIG. 6—*Continued.*

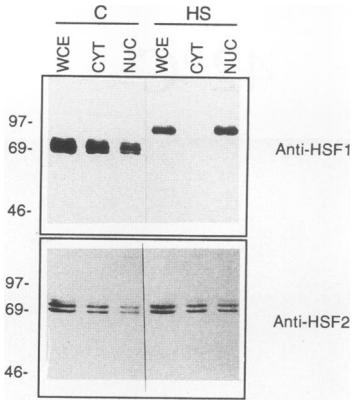

FIG. 7. Biochemical fractionation of HSF1 and HSF2. Whole cell extracts (WCE) and fractionated cytoplasmic (CYT) and nuclear (NUC) extracts from unstressed (C) or heat-shocked (HS) 3T3 cells were subjected to Western blot analysis using the HSF1 antiserum (upper panel) or HSF2 antiserum (lower panel). Equal proportions of cytoplasmic and nuclear extracts were loaded in the indicated lanes.

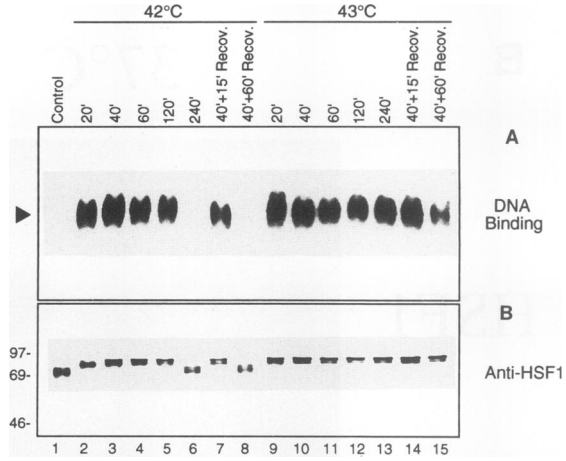

FIG. 8. Correlation of HSF1 DNA binding and phosphorylation state during heat shock, attenuation, and recovery. (A) Extracts from 3T3 cells heat shocked at 42°C for 0 min (control), 20 min, 40 min, 60 min, 120 min, 240 min, 40 min plus 15-min recovery at 37°C, and 40 min plus 60-min recovery at 37°C or heat shocked at 43°C for 20 min, 40 min, 60 min, 120 min, 240 min, 40 min plus 15-min recovery at 37°C, and 40 min plus 60-min recovery at 37°C were subjected to gel shift analysis. The apparent reduction in HSF1 DNA-binding activity in lane 15 was the result of underloading of the sample. (B) Western blot analysis of HSF1 during a heat shock time course. Aliquots of extracts from the heat shock time courses used for the gel shift analysis shown in panel A were subjected to Western blot analysis using the HSF1 antiserum.

phorylated, activated form is localized to the nucleus. Similar results were obtained for HeLa cells (34).

Similar to HSF1, HSF2 is distributed in a diffuse pattern in both the cytoplasm and nucleus of unstressed 3T3 cells (Fig. 6A, lower panel). In contrast to HSF1, the overall intracellular distribution of HSF2 in 3T3 cells is unchanged following heat shock. There are, however, differences in the appearance of the HSF2 staining pattern after heat shock. Diffuse HSF2 staining is observed in the cytoplasm and nucleus of heat-shocked 3T3 cells. In addition, intensely staining punctate structures are observed in the cytoplasm. Western blot analysis confirmed that HSF2 is present in both the cytoplasmic and nuclear fractions of unstressed and heat-shocked 3T3 cells (Fig. 7, lower panel).

In HeLa cells as in 3T3 cells, HSF2 is found in both the cytoplasm and nucleus of unstressed cells, and its intracellular localization is unaffected by heat shock. However, there are differences in the HSF2 staining pattern between 3T3 and HeLa cells. Although there is diffuse staining in the cytoplasm and nuclei of both 3T3 and HeLa cells, multiple punctate, brightly staining structures are also observed in the nuclei of both unstressed and heat-shocked HeLa cells (Fig. 6B, lower panel). There are approximately 20 of these HSF2 structures per cell, compared with 4 to 6 HSF1 granules in heat-shocked HeLa cells. In addition, these structures are smaller than those observed for HSF1, averaging 1 to 1.3 μm.

Correlation of DNA binding, phosphorylation, oligomeric state, and nuclear localization of HSF1 during heat shock, attenuation, and recovery. Previous studies have demonstrated that the rate of hsp70 gene transcription, HSE-binding activity, and in vivo occupancy of the HSEs on the hsp70 promoter are tightly correlated during heat shock of HeLa cells (2, 3, 27). During the attenuation phase of the heat shock response, the decreased rate of hsp70 transcription is correlated with a reduction in HSF DNA-binding activity (3). To determine whether the DNA-binding activity, levels of phosphorylation, oligomeric state, and nuclear localization of HSF1 are correlated during heat shock, attenuation, and recovery, extracts were prepared from 3T3 cells exposed to 42 or 43°C for various times and analyzed by gel shift, Western blotting, and pore exclusion limit electrophoresis. Cells from each time point were also examined by immunofluorescence analysis.

As demonstrated in Fig. 8A, HSF1 DNA-binding activity is induced within 20 min of exposure to a 42°C heat shock (lane 2). Western blot analysis shows that heat-induced phosphorylation of HSF1 also occurs within 20 min (Fig. 8B, lane 2). The heat-induced DNA-binding activity and phosphorylation remain unchanged through 120 min (Fig. 8A and B, lanes 3 to 5). After 240 min of continuous heat shock at 42°C, the DNA-binding activity of HSF1 is no longer observed, and its disappearance is correlated with a decrease in HSF1 phosphorylation to the levels in unstressed cells (compare lanes 1 and 6). In 3T3 cells heat shocked for 40 min at 42°C and allowed to recover at 37°C, HSF1 DNA-binding activity and phosphorylation remain near maximal after a 15-min recovery (lane 7) but return to the levels observed in unstressed cells after 60 min of recovery (lane 8). In cells exposed to a 43°C heat shock, the HSF DNA-binding activity and heat-induced phosphorylation reach maximal levels by 20 min (lane 9) but, in contrast to the 42°C heat shock, persist through 240 min of continuous heat shock (lanes 10 to 13) and even after 60 min of recovery at 37°C (lane 15). Identical results were obtained for human HeLa cells (data not shown).

Pore exclusion limit analysis and chemical cross-linking of extracts from the 42 and 43°C heat shock time courses showed that oligomerization of HSF1 parallels its DNA-binding activity and heat-induced phosphorylation (34). Likewise, immunofluorescence analysis demonstrated that localization of HSF1 to the nucleus in response to heat shock was also correlated tightly with HSF1 DNA-binding activity, heat-induced phosphorylation, and oligomerization (34). These results demonstrate that there is a very tight correlation among HSF1 DNA-binding activity, oligomerization,

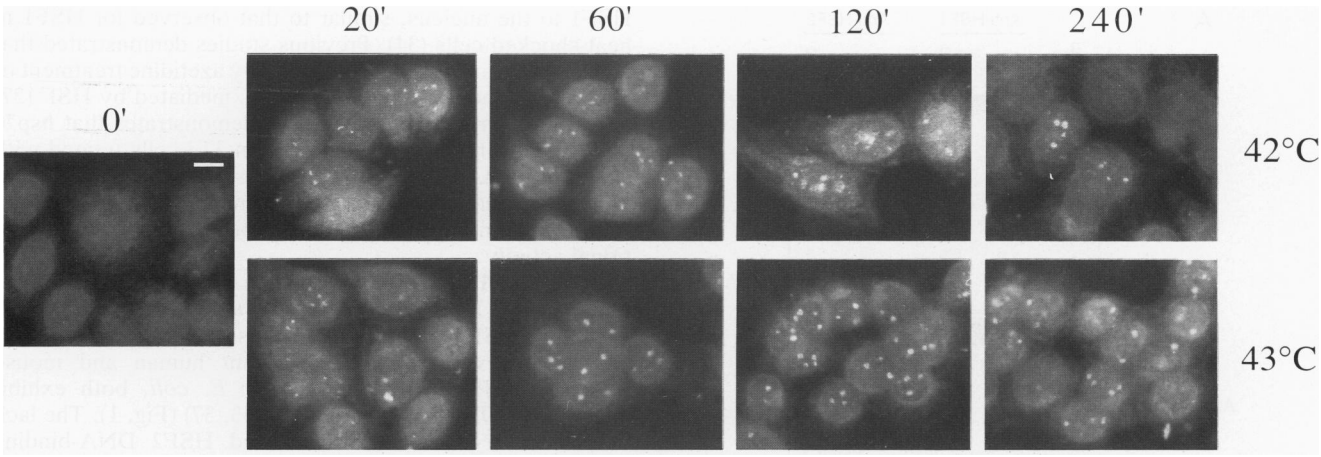

FIG. 9. Kinetics of HSF1 granule formation in heat-shocked HeLa cells. Immunofluorescence analysis was performed on HeLa cells prior to heat shock (0') or after incubation at 42 or 43°C for 20, 40, 60, 120, and 240 min, using the HSF1 antiserum. The bar corresponds to 10 μm.

heat-induced phosphorylation, and nuclear localization, during both activation and attenuation of the heat shock response. These findings suggest not only that these properties are functionally interdependent but also that acquisition of one or more of these properties may be involved in regulating acquisition of the others.

Kinetics of HSF1 granule formation in heat-shocked HeLa cells. In the initial immunofluorescence studies, a punctate staining pattern for HSF1 was observed in the nuclei of heat-shocked HeLa cells. To more thoroughly characterize the appearance of the HSF1 punctate structures in HeLa cells and to determine whether their appearance is correlated with HSF1 DNA-binding activity, immunofluorescence analysis was performed on cells heat shocked at different temperatures for various times in conjunction with gel shift analysis. The results of these analyses demonstrate that the kinetics of granule formation are tightly correlated with HSF1 DNA-binding activity (Fig. 9; 34). The HSF1 punctate structures are observed within 20 min of heat shock at 42°C, although not in every cell. The percentage of cells containing the structures increases during the 42°C heat shock, reaching a peak at between 60 and 120 min. In addition, the granules increase in size, reaching a maximum at approximately 120 min. Granules were still present in cells heat shocked for 40 min at 42°C and allowed to recover for 15 min at 37°C but were completely gone by 60 min of recovery at 37°C (34).

In contrast, the fraction of cells containing granules and the granule sizes increased more rapidly in cells heat shocked at 43°C. The granules reached a maximum size by 20 to 60 min and were present in virtually all of the cells by 60 min. Cells heat shocked for 40 min at 43°C and then allowed to recover at 37°C for 15 min still contained the structures; however, in contrast to cells subjected to the 42°C heat shock, the HSF1 structures in the HeLa cells heat shocked at 43°C persisted for at least 120 min of recovery at 37°C (34). These results show that the size and kinetics of granule formation reflect the severity of the heat stress.

Characterization of HSF1 and HSF2 properties in cells exposed to heavy metals and amino acid analogs. In addition to heat shock, a number of other agents such as heavy metals and amino acid analogs have been shown to induce HSE-binding activity and hsp70 gene transcription (27). To determine whether HSF1 is also the primary component of the HSE-binding activity in cells treated with inducers of the stress response other than elevated temperature, 3T3 cells were treated with either cadmium sulfate or the proline analog azetidine at 37°C for various times. Gel shift analysis demonstrated that maximal levels of HSE-binding activity were observed after 120 min of treatment with cadmium sulfate and 240 min of treatment with azetidine (34). Extracts from these time points were then subjected to gel shift analysis after preincubation with either the HSF1 or HSF2 antiserum. Similar to the results obtained for HSE-binding activity from cells subjected to heat shock (Fig. 3), the HSF1 antiserum recognizes the HSE-binding activity induced in cells treated with cadmium sulfate or azetidine, even at dilutions of up to 1:250 (Fig. 10A). The HSF2 antiserum has an effect on the induced HSE-binding activities only at high concentrations (1:10). The recognition pattern of the cadmium sulfate- and azetidine-induced HSF DNA-binding activity by the HSF1 and HSF2 antisera is essentially identical to that observed for recombinant HSF1 (Fig. 10B). These results demonstrate that the HSF DNA-binding activity induced by cadmium sulfate or azetidine, like that induced by heat, is composed primarily of HSF1.

Our previous analyses demonstrated that HSF1 undergoes a heat-dependent increase in phosphorylation (Fig. 4 and 8). Western blot analysis was performed to determine whether HSF1 in cells treated with cadmium sulfate or azetidine also undergoes an increase in phosphorylation. Figure 10C (upper panel) shows that treatment of cells with cadmium sulfate results in an increase in size of HSF1 similar to that observed for HSF1 in heat-shocked cells. Phosphatase treatment of these extracts indicated that the increase in size of HSF1 is due to phosphorylation (34). In contrast, the size of HSF1 in cells treated with azetidine is identical to that of untreated cells. This result shows that induction of HSF1 DNA-binding activity by azetidine treatment, unlike activation of HSF1 in cells exposed to heat or cadmium sulfate, is not accompanied by an increase in phosphorylation. The size of HSF2 is not affected by treatment of cells with cadmium sulfate or azetidine (Fig. 10C, lower panel).

Results of pore exclusion limit electrophoresis and cross-linking experiments showed that the oligomeric state of HSF1 in cadmium sulfate- and azetidine-treated cells is similar to that of HSF1 in heat-shocked cells (34). Immunofluorescence analysis also showed that treatment of cells with cadmium sulfate or azetidine results in localization of

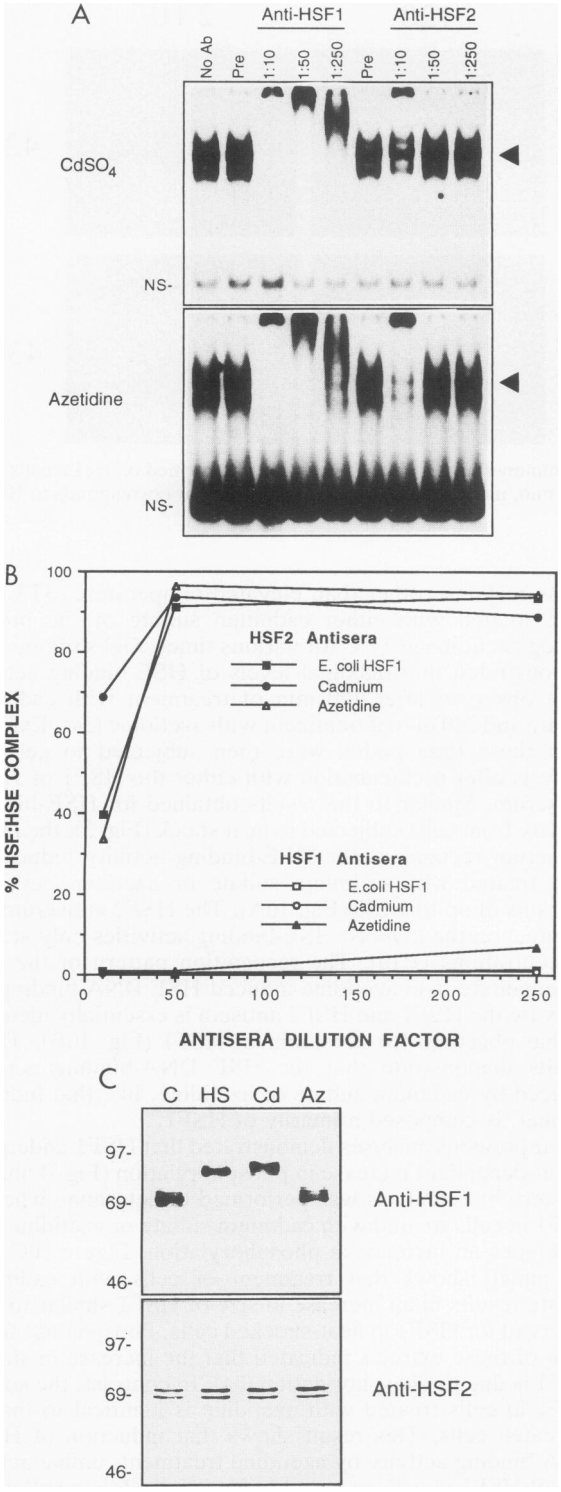

FIG. 10. Analysis of HSF DNA-binding activity and phosphorylation state in 3T3 cells treated with cadmium sulfate or azetidine. (A) Extracts from 3T3 cells treated at 37°C with 30 μM cadmium sulfate for 120 min (upper panel) or 5 mM azetidine for 240 min (lower panel) were incubated with the HSF1 or HSF2 antiserum and subjected to gel mobility shift analysis as described in the legend to Fig. 3. NS, nonspecific complex. (B) Recognition of HSF-DNA complexes from cells treated with cadmium sulfate or azetidine by the HSF1 and HSF2 antisera was quantitated by using a scanning densitometer and plotted as a percentage of HSF-DNA complex present relative to the maximal HSF-DNA complex (no antibody added). Superimposed on these results are those obtained for recombinant bacterially produced HSF1 (Fig. 3). (C) Extracts from unstressed 3T3 cells (C) or cells subjected to heat shock (HS) or treatment with cadmium sulfate (Cd) or azetidine (Az) as described for panel A were subjected to Western blot analysis using the HSF1 antiserum (upper panel) or HSF2 antiserum (lower panel). Sizes are indicated in kilodaltons.

HSF1 to the nucleus, similar to that observed for HSF1 in heat-shocked cells (34). Previous studies demonstrated that hsp70 gene transcription is induced by azetidine treatment of HeLa cells and that this induction is mediated by HSF (27, 55). Run-on transcription analysis demonstrates that hsp70 and hsp90 transcription is induced in 3T3 cells treated with azetidine (34). Taken together, these results indicate that phosphorylation of HSF1 is not essential for its oligomerization, DNA-binding ability, nuclear localization, or transcriptional activity.

HSF1 and HSF2 overexpressed in 3T3 cells are constitutively active. In unstressed mouse and human cells, both HSF1 and HSF2 are present in forms that are unable to bind DNA. However, when recombinant human and mouse HSF1 and HSF2 are expressed in *E. coli*, both exhibit constitutive DNA-binding activity (33, 37) (Fig. 1). The lack of correct regulation of HSF1 and HSF2 DNA-binding activity in bacteria has been speculated to be due to improper protein folding in the prokaryotic system or the absence of a negative regulator which normally functions in higher eukaryotic cells to repress HSF1 and HSF2 DNA-binding activity. These results prompted us to examine whether HSF1 or HSF2 overexpressed in mouse cells would be correctly regulated or whether high levels of these factors may still overcome the negative regulation, perhaps by titrating out a negative regulatory factor, and result in constitutive DNA-binding activity.

To test this hypothesis, expression vectors containing HSF1 or HSF2 under the control of the human β-actin promoter were transfected into mouse 3T3 cells. As a control, cells were transfected with β-Act-Hox1.3, which contains the mouse homeobox gene Hox1.3 under the control of the β-actin promoter. As shown in Fig. 11A (upper panel), Western blot analysis demonstrates that extracts from 3T3 cells transfected with the HSF1 expression vector (lane 5) contain at least three- to fourfold more HSF1 than do extracts from either untransfected, unstressed, or heat-shocked 3T3 cells (lanes 1 and 2), cells transfected with the Hox 1.3 expression vector (lanes 3 and 4), or cells transfected with the HSF2 expression vector (lane 6). Similar results are observed for HSF2 in cells transfected with the HSF2 expression vector (lower panel, lanes 1 to 6). The sizes of HSF1 and HSF2 from transfected cells are identical to those found in unstressed 3T3 cells (compare lanes 1 and 5 in the upper panel and lanes 1 and 6 in the lower panel).

Gel shift analysis was then performed to examine whether the DNA-binding activity of the transfected HSF1 or HSF2 was correctly regulated or whether it was constitutive as it is when expressed in *E. coli*. Figure 11B demonstrates that both HSF1 (lane 5) and HSF2 (lane 6) bind DNA constitutively when overexpressed in 3T3 cells. The HSF DNA-binding activity does not result from activation caused by the transfection process or overexpression of a nuclear-localized protein in these cells, since HSF DNA-binding activity is not observed in extracts of cells transfected with β-Act-neo-

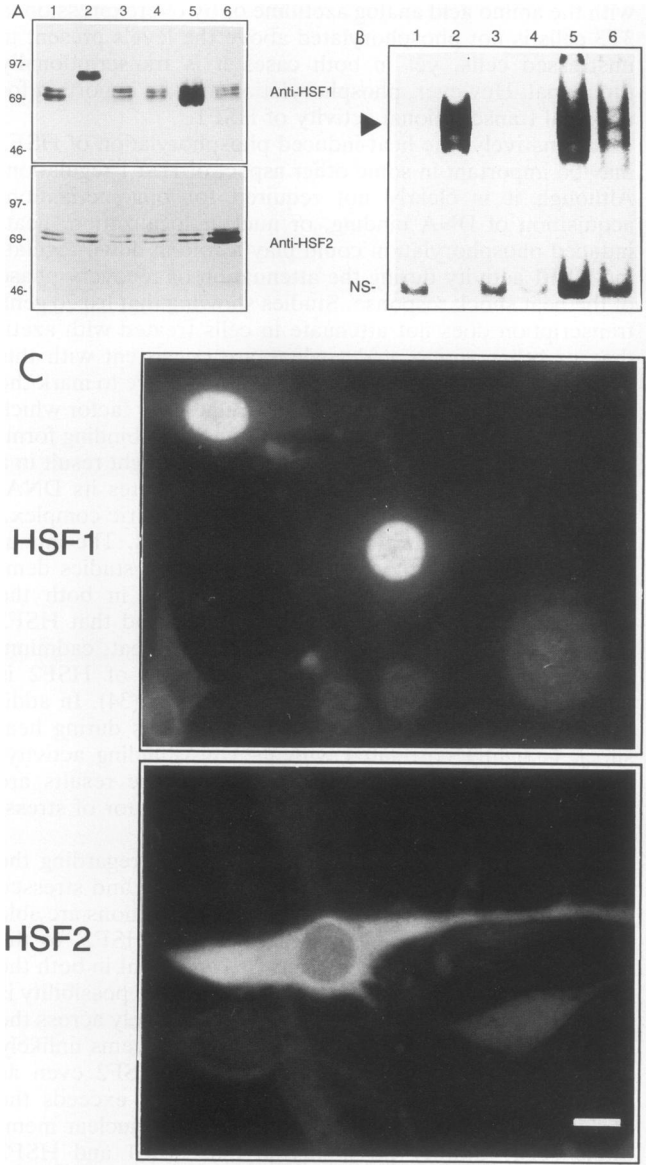

FIG. 11. Evidence that HSF1 and HSF2 overexpressed in 3T3 cells are constitutively active. (A) Extracts from untransfected 3T3 cells and 3T3 cells transfected with the HSF1 or HSF2 overexpression vector were subjected to Western blot analysis using the HSF1 antiserum (upper panel) or HSF2 antiserum (lower panel). Lanes 1 and 2 correspond to extracts from unstressed and heat-shocked 3T3 cells, respectively. The other lanes were loaded with extracts from 3T3 cells transfected as follows: lane 3, with β-Act-Hox1.3; lane 4, same as lane 3 but cells were heat shocked at 42°C for 90 min and allowed to recover at 37°C for 60 min; lane 5, with β-Act-HSF1; and lane 6, with β-Act-HSF2. Sizes are indicated in kilodaltons. (B) Gel shift analysis was performed with extracts from 3T3 cells transfected with HSF1 and HSF2 overexpression vectors. Lanes correspond to those in panel A. Specific HSF-DNA complexes (triangle) are indicated along with nonspecific protein-DNA complexes (NS). (C) Immunofluorescence analysis of 3T3 cells transfected with the HSF1 or HSF2 expression vector was performed by using the HSF1 and HSF2 antisera, respectively. The bar corresponds to 15 μm.

Hox1.3 (lanes 3 and 4). In addition, gel shift analysis in the presence of the HSF1 and HSF2 antisera demonstrated that the HSF DNA-binding activity of extracts from cells transfected with the HSF1 expression vector is composed only of HSF1, while that from cells transfected with the HSF2 expression vector is composed only of HSF2 (34). This result demonstrates that overexpression of HSF1 does not activate the DNA-binding activity of endogenous HSF2 and, conversely, that overexpression of HSF2 does not lead to activation of endogenous HSF1 DNA-binding activity. Cross-linking analysis of extracts from cells transfected with HSF1 or HSF2 expression vector demonstrated that the activated HSF1 and HSF2 both form oligomeric complexes similar in size to those observed for HSF1 in heat-shocked 3T3 cells (34) (Fig. 5).

Immunofluorescence analysis was performed on cells transfected with the HSF1 or HSF2 expression vector to determine the intracellular localization of the overexpressed proteins. Figure 11C shows that HSF1 overexpressed in 3T3 cells is localized exclusively to the nucleus, in a pattern identical to that observed in cells exposed to heat (Fig. 6) and other stresses. The observed nuclear localization of HSF1 does not result from the transfection protocol or from overexpression of a nuclear-localized protein, since untransfected cells in the same field (Fig. 11C) or cells overexpressing murine Hox1.3 (34) have an HSF1 staining pattern identical to that of unstressed cells. HSF2 overexpressed in 3T3 cells, in contrast to HSF1, is found in both the cytoplasm and nucleus of cells transfected with the HSF2 expression vector (Fig. 11C).

To determine whether the overexpressed HSF1 or HSF2 is capable of activating transcription of an HSE-containing promoter in the absence of stress, the HSF expression vectors were cotransfected into 3T3 cells with hsp70-luciferase, which contains the human hsp70 promoter upstream of the firefly luciferase gene, and the construct RSV-CAT as an internal control for transfection efficiency. The relative abilities of overexpressed HSF1 and HSF2 to activate transcription were determined by comparing the luciferase activities from cells overexpressing murine Hox 1.3. In three separate experiments, heat shock of cells cotransfected with hsp70-luciferase and β-Act-Hox1.3 resulted in a mean 3.8 (± 1.2)-fold induction of luciferase activity in comparison with unstressed cells. Cotransfection of the HSF1 expression vector with hsp70-luciferase resulted in a mean 1.8 (± 0.25)-fold-higher luciferase activity relative to cells cotransfected with hsp70-luciferase and β-Act-Hox1.3. Cells cotransfected with the HSF2 expression vector exhibited mean 4.5 (± 1.7)-fold-higher levels of luciferase activity relative to controls.

These results demonstrate that activation of HSF1 DNA-binding activity, oligomerization, nuclear localization, and transcriptional activity can occur in the absence of environmental stress. These findings provide support for a model of regulation in which the activities of HSF1 and HSF2 are repressed by a titratable negative regulatory factor or factors. These results, consistent with the results obtained for azetidine-induced HSF (Fig. 10) (34), indicate that stress-induced phosphorylation of HSF1, although it was found to be correlated tightly with HSF1 DNA-binding activity, oligomerization, and nuclear localization (Fig. 8) (34), is clearly not required for acquisition of any of these properties because HSF1 overexpressed in 3T3 cells is not phosphorylated above the levels observed in unstressed cells and yet is constitutively active.

DISCUSSION

Distinct functional roles of HSF1 and HSF2. The discovery that a number of eukaryotic species, including tomato,

chicken, mouse, and human, all have at least two HSF genes, while yeasts and the fruit fly have a single HSF gene, has prompted questions regarding the functions of these multiple HSFs. We have begun to address this question by determining whether both HSF1 and HSF2 are involved in mediating stress-induced heat shock gene transcription, or whether this function has been segregated to one of these HSFs. To accomplish this, we used antisera which specifically recognize HSF1 and HSF2 to determine the physical, biochemical, and biological properties of these two factors in both unstressed and stressed mouse and human cells.

The results of these studies reveal that HSF1 is the primary activator of heat shock gene transcription in response to stresses such as heat shock, cadmium sulfate, and the amino acid analog azetidine. Furthermore, HSF2 does not appear to be involved in the transcriptional activation of heat shock genes induced by these stresses. These results are consistent with previous findings demonstrating that mouse HSF1 translated in rabbit reticulocyte lysate displays heat-inducible DNA binding (35). In addition, all of the peptide sequences derived from HSF purified from heat-shocked HeLa cells were found to be encoded by the human HSF1 cDNA, while only two of six were found in the predicted human HSF2 protein sequence (33, 37).

Since HSF2 does not appear to be involved in the activation of heat shock gene transcription induced by heat shock, cadmium sulfate, or the amino acid analog azetidine, what is its function? Previous studies revealed that HSF DNA-binding activity and hsp70 gene transcription are activated during hemin-induced differentiation of human K562 erythroleukemia cells (50). Recent work has demonstrated that the HSF DNA-binding activity induced by hemin is composed primarily of HSF2 (39). These results indicate that HSF2 may function to activate heat shock gene transcription during differentiation or other cellular processes.

Physical and biochemical properties of activated HSF1. HSF1 exhibits a number of biochemical properties which are consistent with its role as the mediator of stress-induced heat shock gene transcription. For example, HSF1 exists in unstressed cells predominantly as a monomer but is converted to a large, possibly trimeric complex upon stress treatment (Fig. 5). In contrast, the native molecular weight of HSF2 is not affected by stress treatment (Fig. 5) (34). The tight correlation between HSF1 oligomerization and DNA-binding activity (Fig. 8) (34) suggests that oligomerization may be a prerequisite for HSF1 DNA-binding activity. The heat-induced oligomerization of HSF1 is reminiscent of the hormone-mediated dimerization of steroid receptors such as the estrogen receptor (21). Control of oligomerization may therefore be a common mechanism for the regulation of inducible transcription factors.

Our results show that HSF1 undergoes an increase in phosphorylation in cells exposed to heat or cadmium sulfate (Fig. 4 and 10). Yeast HSF has been shown to undergo phosphorylation during heat shock concomitant with an increase in hsp70 gene transcription, suggesting that HSF phosphorylation functions to enhance its transcriptional activity (45). Our studies demonstrating that during heat shock, phosphorylation of HSF1 is tightly correlated with its DNA-binding ability, coupled with previous studies from our laboratory showing that the DNA-binding activity of HSF from heat-shocked cells parallels transcription of the hsp70 gene (2, 3), suggest that the same may be true for HSF1 in mammalian cells. However, increases in phosphorylation do not appear to be absolutely required for acquisition of HSF1 transcriptional activity, since HSF1 activated by treatment with the amino acid analog azetidine or by overexpression in 3T3 cells is not phosphorylated above the levels present in unstressed cells, yet in both cases it is transcriptionally functional. However, phosphorylation may be important for maximal transcriptional activity of HSF1.

Alternatively, the heat-induced phosphorylation of HSF1 may be important in some other aspect of HSF1 regulation. Although it is clearly not required for oligomerization, acquisition of DNA binding, or nuclear localization, heat-induced phosphorylation could play a role in down-regulating HSF1 activity during the attenuation or recovery phase of the heat shock response. Studies showing that hsp70 gene transcription does not attenuate in cells treated with azetidine or other amino acid analogs are consistent with this possibility (10, 55). Phosphorylation could serve to mark the activated HSF1 for recognition by a regulatory factor which acts to restore it to the monomeric, non-DNA-binding form. Alternatively, removal of these phosphates might result in a conformational change in HSF1 that inactivates its DNA-binding activity and/or dissociates the oligomeric complex.

Intracellular localization of HSF1 and HSF2. The immunofluorescence and biochemical fractionation studies demonstrate that HSF1 and HSF2 are present in both the cytoplasm and nucleus of unstressed cells and that HSF1 relocalizes to the nucleus after exposure to heat, cadmium sulfate, or azetidine, while the localization of HSF2 is unaffected by stress treatment (Fig. 6 and 7) (34). In addition, the localization of HSF1 to the nucleus during heat shock is tightly correlated with its DNA-binding activity, oligomeric state, and phosphorylation. These results are consistent with the role of HSF1 as the mediator of stress-induced heat shock gene transcription.

These results raise a number of questions regarding the localization of HSF1 and HSF2 in unstressed and stressed cells and the mechanism by which stress conditions are able to effect a redistribution of HSF1, but not HSF2, to the nucleus. First, why are HSF1 and HSF2 present in both the cytoplasm and nucleus of unstressed cells? One possibility is that HSF1 and HSF2 are able to equilibrate freely across the nuclear membrane of unstressed cells. This seems unlikely given that the native sizes of HSF1 and HSF2 even as monomers would be at least 70 kDa, which exceeds the exclusion limit for free diffusion through the nuclear membrane (11). Another possibility is that HSF1 and HSF2 polypeptides may be transiently associated with other proteins which may shuttle some of the HSF1 and HSF2 molecules into the nucleus. Recent work suggests that 70-kDa heat shock proteins are required for the translocation of nucleoplasmin into the nucleus (38). Perhaps hsp70 is also involved in the translocation of HSF1 and HSF2.

How does HSF1 become concentrated in the nucleus when the cell is subjected to stress? Two related hypotheses may provide an explanation for this phenomenon. First, HSF1 may contain a cryptic nuclear localization signal which is unmasked as it becomes activated and directs the HSF1 in the cytoplasm to translocate into the nucleus. However, an examination of the amino acid sequences for mouse HSF1 and HSF2 reveals no obvious motifs analogous to known nuclear localization signal sequences. Alternatively, HSF1 may contain a cryptic nuclear retention signal which is unmasked by the activation process. For example, as HSF1 becomes activated and acquires DNA-binding activity, it could be sequestered to the nucleus by binding to target sequences in the chromatin.

Recent studies using antibodies directed against *Drosophila* HSF indicated that HSF is localized exclusively in

the nucleus of both unstressed and heat-shocked *Drosophila* S2 cells (53). The reason for the difference between these results and our own is unclear but may simply reflect differences in the localization properties of HSF in *Drosophila* versus mouse and human cells. In light of these differences, it will be important to determine the subcellular localization of HSF in a number of other species to determine whether there are any generalities of HSF localization properties in divergent species.

The functional significance of the brightly staining granules that contain HSF1 and HSF2 in HeLa cells is unclear. The fact that we have observed these structures in a number of human cell lines (T98G glioblastoma and Y79 neuroblastoma cells) and not in any of several mouse cell lines that we have studied suggests that this may be a characteristic difference between human and mouse cells (34). The kinetics of appearance of the HSF1 granules in the nuclei of HeLa cells during heat shock correlates very well with the kinetics of HSF DNA binding and heat shock gene transcription in HeLa cells (Fig. 8 and 10) (3, 27, 34). These particles may represent foci of HSF1 and HSF2 binding to target genes in the chromatin. This interpretation is consistent with results for *Drosophila* cells, in which staining of specific bands in the polytene chromosomes with HSF antisera indicates that HSF is localized to specific chromosomal loci (53). Alternatively, the granules may represent large aggregate particles of inactive HSF1 and HSF2, though the correlation between the appearance of the HSF1 granules and HSF1 DNA-binding activity would argue against this. Studies using HSF1 or HSF2 immunofluorescence in conjunction with in situ hybridization using DNA probes specific to heat shock genes would be useful in addressing these possibilities.

Nuclear structures similar to the granules observed with the HSF1 and HSF2 antisera have been seen previously in immunofluorescence studies using antibodies directed against other proteins. *Drosophila* HSF is localized in granular structures in the nucleus of both unstressed and heat-shocked cells (53). Nuclear structures with a speckled appearance have also been observed in immunofluorescence studies using antibodies raised against small nuclear ribonucleoproteins (7, 13, 40, 41, 46). These speckles correspond to spliceosomes and represent the sites of the RNA splicing machinery (7).

Negative regulation of HSF1 and HSF2 activities. HSF1 and HSF2 are present in unstressed cells in an inactive, non-DNA-binding state which can be activated under certain conditions to oligomerize, bind DNA, and transcriptionally activate heat shock gene expression. However, recombinant *Drosophila* HSF and human and mouse HSF1 and HSF2 expressed in *E. coli* bind DNA constitutively (8, 33, 37) (Fig. 1). It has been speculated that the lack of proper regulation of HSF DNA-binding activity in bacteria is due to either improper folding of the overexpressed HSF or the lack of a negative regulatory factor in *E. coli* that is present in eukaryotic cells (8, 33). Our results showing that mouse HSF1 and HSF2 overexpressed in mouse cells are constitutively active in the absence of stress provide support for the existence of titratable negative regulatory factors which function to keep HSF1 and HSF2 in an inactive state (Fig. 11). At least for HSF1, the negative regulatory factor does not appear to exert its control by maintaining a stable complex with its target because HSF1 exists predominantly as a monomer in unstressed cells (Fig. 5). This possibility does exist for HSF2, however, which exhibits a native molecular weight that is consistent with interaction either with itself or with other protein(s).

Although both HSF1 and HSF2 are under negative control in unstressed cells, each is activated by different types of signals. This raises the question of whether HSF1 and HSF2 are under the control of the same or different negative regulatory factors. Our results showing that HSF1 is activated by stress treatments while HSF2 is not, and the results of previous work demonstrating that HSF1 translated in vitro in rabbit reticulocyte lysates binds DNA only after heat treatment, while HSF2 DNA-binding activity is constitutive, suggest that the regulatory molecules controlling each factor may be different. In addition, activation of HSF2, but not HSF1, by hemin treatment of human K562 cells (39) is consistent with the idea of different negative regulators. If, on the other hand, the activities of both HSF1 and HSF2 are in fact regulated by a single regulatory factor, then distinct signal transduction pathways must have evolved to selectively control the activity of the negative regulator with respect to each of the two HSFs.

Work from a number of systems and laboratories strongly suggests that at least in the case of HSF1, the regulatory molecules are in fact the heat shock proteins themselves. Recent studies indicate that hsp70 is associated with HSF1 during heat shock (1, 6). Addition of exogenous hsp70 to cytoplasmic extracts prevents in vitro activation of HSF (1). These results, taken together, suggest that hsp70 may regulate the activation as well as the attenuation phase of the heat shock response in human cells. A large number of studies of *E. coli* have demonstrated that the bacterial heat shock proteins DnaK, DnaJ, and GrpE interact with and regulate the levels and activity of the bacterial heat shock-specific σ^{32} (47, 48, 51). The bacterial heat shock proteins regulate σ^{32} through direct protein-protein interactions (14, 23).

The results of our experiments have elucidated some of the aspects of the regulation of HSF1 and HSF2 activity in mammalian cells and have identified HSF1 as the mediator of the stress response. Fundamental questions remain to be solved, especially with respect to the negative regulatory mechanisms which control the activity of HSF1 and HSF2. Studies which extend this work by utilizing co-overexpression of wild-type or mutant HSF1 or HSF2 with hsp70 or other potential negative regulatory molecules in *E. coli* and mammalian cells should provide some of the answers to these questions.

ACKNOWLEDGMENTS

K. D. Sarge and S. P. Murphy contributed equally to this work.

We especially thank Ok-Kyong Park-Sarge for assistance in immunizing rabbits. We thank Sue Fox for expert photography and Jim Sullivan for advice on photomicroscopy. We also thank Benette Phillips, Barbara Wu, and members of our laboratory for critical review of the manuscript.

These studies were supported by grants from the National Institutes of Health and March of Dimes and by an American Cancer Society Faculty Research Service award to R.I.M. and Individual National Research Service awards to K.D.S. and S.P.M.

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